Center for Veterinary Biologics
and
National Veterinary Services Laboratories
Testing Protocol

Supplemental Assay Method for Potency Assay of *Leptospira interrogans* Serovar *icterohaemorrhagiae* Bacterins

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Supplemental Assay Method for Potency Assay of *Leptospira interrogans* Serovar *icterohaemorrhagiae* Bacterins

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1. Introduction

1.1 Background

This Supplemental Assay Method (SAM) describes the hamster vaccination-challenge method used to determine potency of *Leptospira icterohaemorrhagiae* bacterins as prescribed by the Code of Federal Regulations, Title 9 (9 CFR), Part 113.102.

1.2 Keywords

Leptospira icterohaemorrhagiae, hamster, potency, 9 CFR 113.102, vaccination-challenge

2. Materials

2.1 Equipment/instrumentation

2.1.1 Microscope with darkfield capability

2.1.2 Forceps, 5½ in, rat-tooth

2.1.3 Dissecting pins, 1-1½ in

2.1.4 Necropsy board, wooden or equivalent

2.1.5 Tissue grinder, 15 ml, TenBroeck or equivalent

2.2 Reagents/supplies

2.2.1 Syringes, 1 ml tuberculin and 3 ml

2.2.2 Needles, 25 ga x 5/8 in, 20 ga x 1½ in

2.2.3 Disposable scalpels, size 21

2.2.4 Glass screw-top tubes, 20 x 150 mm

2.2.5 Pipettes, 10 ml and 25 ml, cotton-plugged

2.2.6 Serum bottles, 10 ml and 30 ml, with rubber stoppers and aluminum ring seals

2.2.7 Cotton balls

2.2.8 Microscope slides, 25 x 75 mm

2.2.9 Cover glass, 22 x 22 mm

2.2.10 1% Bovine Serum Albumin Diluent (BSAD)

2.2.11 0.85% saline solution

2.2.12 Ethyl alcohol, 70% v/v

2.2.13 *L. icterohaemorrhagiae* challenge culture, hamster-virulent

2.2.14 Water, distilled or deionized, or water of equivalent purity

2.3 Animals

2.3.1 Hamsters, adult, 50-90 g. Ten hamsters are required for each bacterin tested. Thirty additional hamsters are required for nonvaccinated controls and LD_{50} titration.

2.3.2 The hamsters must be obtained from the same source and colony. Use either all male or all female hamsters for any 1 test.

2.3.3 House and feed all hamsters in an identical manner.

3. Preparation for the test

3.1 Personnel qualifications/training

Technical personnel need a knowledge of the use of general laboratory chemicals, equipment, and glassware and must have specific training and experience in the safe handling of live *Leptospira* spp. Personnel need specific training in the care and handling of laboratory hamsters. Personnel must have training in the performance of this protocol.

3.2 Preparation of equipment/instrumentation

3.2.1 Operate all equipment according to manufacturer's instructions.

3.2.2 Sterilize all glassware before use.

3.2.3 Use only sterile supplies (pipettes, syringes, needles, rubber stoppers, etc.).

3.3 Preparation of reagents

3.3.1 1% Bovine Serum Albumin Diluent (National Veterinary Services Laboratories [NVSL] media 10119)

Na_2PO_4	0.664 g
KH_2PO_4	0.087 g
Bovine serum albumin, fraction V	10 g
Water	q.s. 1.0 L

Mix until dissolved. If necessary, adjust pH to 7.5. Sterilize by filtration, using a 0.22-µm filter. Store at room temperature (20-25°C) no longer than 3 mo.

3.3.2 0.85% saline (NVSL media 30201)

NaCl	8.5 g
Water	q.s. 1.0 L

Autoclave at 121°C for 15 min. Store at room temperature no longer than 6 mo.

3.4 Preparation of the sample

3.4.1 Shake each bacterin to mix contents thoroughly.

3.4.2 Disinfect the top of the bacterin container with a cotton ball soaked in 70% ethanol.

3.4.3 Dilute each bacterin with saline so that 1 hamster dose (0.25 ml) is equivalent to 1/80 of the recommended host-animal dose.

1. For 2-ml-dose products, dilute the bacterin 1:10 in normal saline (1.0 ml bacterin + 9.0 ml saline).

2. For 5-ml-dose products, dilute the bacterin 1:4 in normal saline (1.0 ml bacterin + 3.0 ml saline).

4. Performance of the test

4.1 Vaccination of hamsters

4.1.1 For each bacterin to be tested, vaccinate 10 hamsters with 0.25 ml of appropriately diluted bacterin (see **Section 3.4.3**), using the route recommended by the manufacturer. If the recommended vaccination route is intramuscular, or if the product is labeled for either intramuscular or subcutaneous use, vaccinate the hamsters intramuscularly in the hind leg. If the label limits administration of that product to the subcutaneous route, vaccinate the hamsters subcutaneously in the abdominal area. For all vaccinations, use a 1.0-ml syringe fitted with a 25-ga x 5/8-in needle.

4.1.2 Retain 10 nonvaccinated hamsters as controls. House and feed control hamsters in a manner identical to the vaccinates.

4.1.3 Retain 20 nonvaccinated hamsters to determine the LD_{50} of the challenge inoculum.

4.1.4 Challenge all hamsters with a virulent suspension of *L. icterohaemorrhagiae* 14 to 18 days after vaccination.

4.2 Preparation of the challenge inoculum

4.2.1 Use homogenized liver tissue from a hamster infected with *L. icterohaemorrhagiae* as the source of the challenge inoculum. Select a clinically ill (preferably moribund) hamster from a group of hamsters that were infected 3-4 days previously with

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L. icterohaemorrhagiae (see current version of BBSOP0061, Storage and Propagation of *Leptospira* serotypes).

4.2.2 Euthanize the hamster with CO_2 as described in the current version of ACUCSOPA101. Pin the dead hamster to a posting board (ventral aspect up), and disinfect the skin with 70% ethanol.

4.2.3 Using aseptic technique, reflect the abdominal skin. Reflect the abdominal musculature to expose the abdominal viscera. Discard the instruments used to open the abdomen.

4.2.4 Using fresh instruments, aseptically remove approximately 1 g of liver tissue. Place liver in a sterile glass tissue grinder. Add 9 ml of sterile BSAD to the grinder. Thoroughly homogenize the liver, taking care to avoid foam formation. This suspension is considered the 1:10 dilution.

4.2.5 Prepare 6 additional serial tenfold dilutions (10^{-2} through 10^{-7}) of the tissue suspension in BSAD (1.0 ml suspension + 9.0 ml diluent). Hold the dilutions at room temperature (20-25°C) no longer than 1 hr before use.

4.2.6 As a check on the density of the organisms in the suspensions, place 2 drops of the 10^{-5} dilution on a microscope slide, cover with a coverslip, and examine under a 200X objective with a darkfield microscope. The 10^{-5} dilution should have approximately 4-20 organisms per field.

4.2.7 If the 10^{-5} dilution has 4-20 organisms per field, the 10^{-7} dilution is usually sufficient to deliver the required challenge of 10-10,000 LD_{50}.

Note: If the 10^{-4} dilution contains >20 organisms per field, dilute the challenge with BSAD until numbers of organisms are appropriate. Examine the 10^{-3} dilution if <4 organisms are present per field in the 10^{-4} dilution; if the 10^{-3} dilution contains the required number of organisms, challenge with the 10^{-5} dilution.

If the 10^{-3} dilution contains <4 organisms per field, select another clinically ill hamster from **Section 4.2.1** and prepare another challenge inoculum that more closely matches the desired organism density. dilution. If the 10^{-4} dilution does not contain at least 4 organisms per field, select another clinically ill hamster from **Section 4.2.1** and prepare another challenge inoculum that more closely matches the desired organism density.

4.2.8 Prepare 4 additional tenfold dilutions beyond the dilution selected for the challenge inoculum. Retain these dilutions to determine the LD_{50} of the challenge inoculum.

4.3 Challenge of test hamsters

4.3.1 Within 1 hr after preparation, inject intraperitoneally (IP) 0.2 ml of the tissue suspension selected in **Section 4.2.7** into each of the vaccinated hamsters and 10 nonvaccinated control hamsters. Use a 1.0-ml syringe fitted with a 25-ga x 5/8-in needle.

4.3.2 For each of the dilutions prepared in **Section 4.2.8**, inject 5 hamsters (0.2 ml, IP). These 4 groups of hamsters will be used to calculate the LD_{50} of the challenge.

4.3.3 Disinfect all work surfaces with 70% ethyl alcohol. Sterilize all contaminated equipment and supplies in the autoclave.

4.4 Observation of hamsters after challenge

4.4.1 Observe all hamsters daily for 14 days following challenge. Record deaths.

4.4.2 At the end of the 14-day observation period, count the remaining hamsters and record results. instruct the animal caretaker to euthanize remaining hamsters.

4.4.3 Calculate the LD_{50} of the challenge inoculum using the Reed-Muench or Spearman-Karber method of calculation.

5. Interpretation of the test results

5.1 Interpret the results as described in 9 CFR, Part 113.102.

5.2 If 8 or more controls die and the hamsters received a challenge of 10-10,000 LD_{50}, the test is valid. Evaluate the results according to the following table.

Stage	Number of Vaccinates	Cumulative Number of Vaccinates	Cumulative Total Dead Hamsters for Satisfactory Serial	Cumulative Total Dead Hamsters for Unsatisfactory Serial
1	10	10	2 or less	5 or more
2	10	20	5 or less	6 or more

5.3 If 3 or 4 vaccinates die in the first stage test, conduct a second stage test in a manner identical to the first stage. If the second stage is used, evaluate each serial according to the second part of the table. Serials pass or fail on the basis of cumulative results.

6. Report of test results

Report test results as described in the current version of BBSOP0020.

7. References

Code of Federal Regulations, Title 9, Part 113.102, U.S. Government Printing Office, Washington, DC, 1999.

8. Summary of revisions

This document was rewritten to meet the current NVSL/CVB Quality Assurance requirements, to clarify practices currently in use in the CVB-L, and to provide additional detail. No significant changes were made from the last protocol.